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SEASONAL CHANGES IN TOTAL AND SOLUBLE OXALATES IN LEAF BLADES AND PETIOLES OF RHUBARB[1]

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PURPOSE OF THE STUDY

The edibility of the leafstalks and the nonedibility of the leaf blades of rhubarb (*Rheum* spp.) are related in general to their content of soluble oxalic acid. The analyses reported in the literature, however, were not made on leaves of known ages. To help establish the best time to harvest rhubarb for market and the range of oxalic acid content, leaves of known age from plants growing at the Arlington Experiment Farm, Rosslyn, Va., were analyzed in 1932 and 1935. The data obtained are also of general biochemical and physiological interest. In the course of the study it was found necessary to evaluate, modify, and improve the method used by Bau (*3*, *4*)[3] for the determination of oxalic acid in plant tissues.

SOME EARLIER STUDIES

In 1886 Berthelot and André (*5*) found that in young plants of *Rumex acetosa* the oxalic acid content of the leaf blade exceeded that of the petioles, or leafstalks, and larger veins by a 12:10 ratio. In old plants the oxalic acid content of the leaf blade was lower than that of petioles and stems with a ratio of 4 to 5. In the leaf blade the acid was predominantly soluble in young and old plants, while in the petioles it was mainly soluble in the young ones and chiefly insoluble in the old ones.

In 1917 Steinmann (*16*) determined the titratable acidity of the petiole and leaf blade of rhubarb at various stages of maturity. He studied the effect of light and darkness on acid content and found an increase in acidity during a day's exposure to sunlight and a progressive decrease in leaves kept in darkness. There was a descending gradient of titratable acidity from leaf mesophyll to veins to petiole.

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[3] Italic numbers in parentheses refer to Literature Cited, p. 46.

Steinmann found evidence that the acid, like carbohydrates, was manufactured in the leaf tissue and that translocation downward followed; there was an increase in acid from the apex to the base of the leaf blade and from the veins to the petiole.

In 1917 Van Itallie and Lemkes (*7*) apparently found that rhubarb leaves contained from 0.30 to 1.11 percent of anhydrous oxalic acid while the petioles contained 0.44 to 0.99 percent. The toxic dose for man had been previously reported as 2 to 5 gm. of oxalic acid, but these writers believed it to be much lower. They noted one fatal case of poisoning from eating the green leaves.

In 1920 Angerhausen (*2*), using Bau's method of analysis (*4*), found that oxalic acid did not occur in rhubarb as oxalic acid but as soluble potassium acid oxalate to which the poisonous properties were due. He stated that potassium acid oxalate reacts with the blood and nerves and robs the latter of calcium. He found that the stalks contained 0.25 percent of soluble oxalate and the leaves 0.50 percent. He reported also that the oxalates in the petiole were almost if not wholly calcium oxalate.

Maue (*10*) in 1920 studied the constituents of rhubarb leaves in order to explain the fatal poisoning and sickness resulting when they were eaten as food. He and five other men ate cooked rhubarb leaves without the slightest ill effects. His analysis of the leaves showed 0.368 percent of soluble oxalate on the green-weight basis. After a physiological experiment he decided that the gastric juice of the stomach would not render the insoluble oxalates soluble. Maue believed that the illness and death sometimes resulting from the consumption of rhubarb leaves was possibly due to a saponin.

Later Ruhland and Wetzel (*14, 15*) found that the youngest petioles contain mostly succinic and malic acids and scarcely a trace of oxalic acid. As the age of the tissue increased there was a sharp decrease in the first two acids and a rapid increase in oxalic acid. These writers believed the high acid content to be a result of deamination of proteins rather than a partial oxidation of carbohydrates. They gave no description of their methods of identification or determination.

Culpepper and Caldwell (*6*) in 1932 determined the seasonal changes in titratable acidity of rhubarb leaves and petioles and found that the maximum total acid content is accompanied by most rapid cell division and increase in size in the young leaf as is the case with young fruits. The acid content of mesophyll tissue was consistently lower than that of the petiole throughoutth e season. The titratable acidity was greatest at the time of full photosynthetic efficiency.

In 1937 Pucher, Clark, and Vickery (*11, 12*) investigated the organic acid composition of rhubarb. They found that the leaf contained *l*-malic, oxalic, and citric acids together with acids of unknown nature. According to these workers (*12, p. 616*), "the composition differs in different parts of the leaf and is profoundly influenced by the age of the leaf and by the season in which it has developed." Their data showed the predominating acid of the petiole to be *l*-malic with oxalic in second place; citric and unknown acids were present in small amounts. In the younger leaf blades they found the unknown acids to predominate with oxalic acid next in amount. In leaf blades developed late in the season oxalic acid predominated over the un-

known acids and *l*-malic and citric acids were present in small amounts. Their data on concentration showed that oxalic, citric, and unknown acids occurred in a gradient that increased from petiole to veins to blade. They were interested more in shifts in composition of the mixtures of the different organic acids in young leaves as compared with old ones than they were in any detailed change in a specific organic acid.

Allsopp (*1*) in 1937 made an extensive study of seasonal changes in the organic acids of rhubarb. He estimated the seasonal changes in actual content of acid in the whole plant by referring the acid content in milligram equivalents to the original piece of resting rhizome from which the plant developed. He also gave the milligram equivalents on a fresh-weight basis (or percentage × 1,000). He analyzed rhizome and shoots (leaves) sampled once a month for 12 months and found marked changes in concentration of malic, citric, oxalic, and unknown acids. His data showed no increase in the amount of any of these acids on sprouting of the rhizome. All of them increased continuously in the leaves during the season of active photosynthesis, suggesting an origin due to photosynthesis or an indirect one from carbohydrates. Also, acids increased in the rhizome during the summer, indicating translocation from the leaves throughout the period of acid formation. Allsopp showed an initial fall in oxalic acid with the growth of the young leaves, after which this acid increased continuously. He reasoned that the marked changes in acidity, particularly the decreased content of all the rhizome acids during the winter, indicated that acids play an active role in metabolism and are not merely passive end products as some workers have suggested.

MATERIAL AND METHODS

MATERIAL

The material on which these studies were based consisted of a 5-year-old planting of the Ruby variety of rhubarb grown at the Arlington Experiment Farm, Rosslyn, Va. This material was identified by the late D. N. Shoemaker as *Rheum hybridum* Murr. This is a species of somewhat uncertain botanical status, but there is no reason to doubt that conclusions based on the study of it would apply also to the common cultivated rhubarb (*R. rhaponticum* L.). The growth and development of the planting as a whole were very good for the climatic conditions that prevail in the district.

METHOD OF SAMPLING

In the first part of the 1932 growing season a series of samples was taken in which every leaf included had been tagged by the time it reached a height of 5 to 7 cm. Leaves were tagged April 11 to 23, and samples were taken 7, 16, 26, 35, 46, and 60 days after the tagging during the period April 18 to June 22. The age of the 70-day-old sample (oldest), however, was estimated; the leaves were selected on July 5 from the healthiest plants and by all signs were the oldest ones in the clump. The blades of practically all these leaves were turning brown and drying at the margin.

The leaves were harvested by pulling them from the crown, as is customary in harvesting rhubarb. The material for analysis was

collected about 10 a. m. on each sampling date, and 30 leaves were harvested for each chemical sample.

The petioles were thinly sliced transversely with a rotary slicer, the mesophyll tissue of the blade was separated from the midrib and most of the large veins, and the material (petiole and leaf-blade tissue separately) was then weighed and immediately put in a fan-driven drier operated at 65° to 70° C.

In 1935 samples were taken from the same plot by the same general procedure, except that tagging dates were scattered throughout the season.

GROWTH MEASUREMENTS

The petiole and leaf-blade lengths of 25 leaves of each sample were measured to the nearest 0.5 cm. at the time of sampling.

DETERMINATION OF TOTAL DRY MATTER

After the green weight was determined, the samples were immediately put in a fan-driven drier at 65° to 70° C., kept there overnight, then removed, further dried in a vacuum oven for 24 hours at 75° and 27 inches of mercury, and finally weighed from a desiccator to determine the total dry matter. They were then ground in a porcelain ball mill and stored in tightly stoppered brown bottles.

BAU'S METHOD FOR OXALIC ACID DETERMINATION

Bau's method of determining oxalic acid (*4*) involves the precipitation of oxalic acid with a buffer precipitating reagent of calcium acetate, which is called "Kalkessig." It is prepared in the following manner: 330 gm. of crystallized sodium acetate is dissolved in 300 ml. of warm water. After cooling, it is filtered into a 500-ml. volumetric flask. To another 500-ml. flask is added a solution of 25 gm. of crystallized calcium chloride in 50-percent acetic acid; finally enough 50-percent acetic acid is added to make up the volume to the 500-ml. mark. Equal volumes of these two solutions are mixed and allowed to stand at 7° C. or below for 48 hours so that traces of calcium oxalate can deposit. After filtering, this solution is ready for use.

Bau's method for determining oxalic acid in plant tissue is as follows: 15 gm. of fresh plant material is placed in a jar or flask filled with carbonic acid (CO_2+H_2O), and 15 ml. of fuming hydrochloric acid is added to destroy the enzymes and plasmolyze the cells. One hour is allowed for cooling. Then approximately 280 ml. of recently boiled distilled water saturated with CO_2 is added to the flask; the mixture is agitated frequently for about 14 hours; then the extracting solution is filtered through a hard filter (Schleicher and Schüll No. 602). For precipitation of oxalic acid the clear solution should not contain more than 0.2 percent of it. An aliquot of this solution is then treated in the cold with one-fifth of its volume of the precipitating reagent, set aside for 38 to 40 hours at 7° C., and filtered through a quantitative filter. The filtrate is measured, and the volume of the wash water is also noted. The precipitate is washed with cold water until the chloride test is negative. The paper and precipitate are ashed in a platinum crucible, weighed as the oxide, dissolved in 0.1 N hydrochloric acid, and titrated back with 0.1 N sodium hydroxide. For the calculation of oxalic acid: 1 ml. of 0.1 N acid=4.40 mg.

MODIFIED AND IMPROVED METHOD FOR OXALIC ACID DETERMINATION

As stated, the Bau method converts calcium oxalate to the oxide, which is weighed, then dissolved, and titrated. In contrast, the improved method converts calcium oxalate to calcium carbonate, which is the final compound to be weighed. Calcium carbonate is a more satisfactory compound to weigh than calcium oxide, because it is not appreciably hygroscopic.

Bau's method can be simplified, because under certain well-defined conditions the solubility of calcium oxalate in the filtrate and in the wash water can be neglected. Also, as stated on page 40, the recovery of oxalic acid from known solutions is not affected by a fairly wide range in pH; this fact is added assurance that the method applies to moderately great changes in acidity.

All the analyses reported in this study were made on dried rhubarb tissue ground to pass a 30-mesh screen. This was found by experiment to be a prerequisite for complete extraction under the conditions of this investigation. Trial analyses were made by Bau's method (ashing to the oxide), but replicate determinations gave such poor agreement in contrast to those by the improved method, whose precision is shown in table 1, that all the analyses reported in this paper were made by the improved method.

Description of Improved Method

Dry tissue ground fine enough to pass a 30-mesh screen is weighed out exactly to 1.25, 2.50, or 4.00 gm. (depending on the oxalate content of the tissue) either by the moisture-free method or by some other whereby the moisture is known. The sample is transferred quantitatively into a 250-ml. volumetric flask and made up to about 225 to 230 ml. with approximately 0.25 N hydrochloric acid. This is then heated on a water bath at 70° C. for 45 to 60 minutes, cooled to 25°, and made up to volume with 0.25 N hydrochloric acid. After a thorough shaking, the extract is filtered twice through the same folded filter. If the filtrate is not clear, hydrochloric acid is added to it to precipitate the plant colloids and the mixture is filtered again on a separate filter (usually necessary only in the determination of soluble oxalic acid). Aliquots are immediately taken from this filtrate for determination of total oxalic acid. One or two 100-ml. aliquots are taken from the clear filtrate, put in 400-ml. beakers, and heated just to boiling. Then 20 ml. (or one-fifth the volume of the aliquot) of the calcium acetate reagent is added slowly while stirring to precipitate the oxalate. The mixture is allowed to stand overnight in a refrigerator at 2° to 7°, and upon removal it is filtered immediately through a prepared asbestos mat in a previously ignited and tared Gooch crucible. Any suitable type of filtering crucible will serve provided it will stand a temperature of 550°.

The precipitate is collected in the weighed crucible with the aid of gentle suction and a rubber policeman. It is necessary to wash it 15 to 20 times with small portions of ice-cold distilled water until it gives a negative chloride test. The volume of the total washings should amount to at least 50 to 60 ml.

The precipitate is dried at 100° C. for about an hour and ignited at

500°±25° in a controlled or calibrated electric muffle furnace for about 2½ hours, whereby the calcium oxalate is converted to calcium carbonate. (This conversion is now a preferred procedure for the gravimetric determination of calcium. Willard and Boldyreff (*17*) have shown this reduction from the oxalate to the carbonate to be quantitative.) The residue, in the form of calcium carbonate,[4] is ready to weigh after it has been cooled in a desiccator. Milligrams of calcium carbonate multiplied by 0.8995 are equivalent to milligrams of anhydrous oxalic acid.

To determine water-soluble oxalic acid in the tissue, distilled water is used instead of 0.25 N hydrochloric acid for the extraction of the oxalates. Because of the presence of pectin and other colloids, it is necessary to make the aliquots 0.25 N with hydrochloric acid, in order to get large and easily filterable calcium oxalate crystals consistently.

Preparation of Precipitating Reagent

By the modified method the oxalic acid is precipitated as the calcium salt by a reagent similar to the one recommended by Bau (*4*), which he called "Kalkessig." It is prepared as follows:

Solution A.—300 gm. of crystalline sodium acetate is dissolved in warm water, cooled, and made to a final volume of 500 ml.

Solution B.—25 gm. of anhydrous calcium chloride is dissolved in distilled water, 50 ml.[5] of 50-percent acetic acid is added, and the mixture is made up to a final volume of 500 ml. with distilled water.

Solution A is mixed with solution B, filtered, set in a refrigerator at 2° to 5° C. for 48 hours, and again filtered. The reagent maintains the hydrogen-ion concentration of the precipitating medium at about pH 5.0 to 5.5. Adding 1 part "Kalkessig" to 5 parts of the solution to be tested was found by experiment to be satisfactory and was used throughout the analyses.

Preparation of Asbestos

The asbestos used in preparing the mats for the Gooch crucibles is thoroughly digested with hydrochloric acid, sodium hydroxide, and nitric acid for several weeks for each treatment. The mats are then washed thoroughly with distilled water to get rid of the fine asbestos that would be washed out and cause a weight loss during subsequent filtration, ignition, and weighing.

Ashing Technique

A platinum-rhodium thermocouple and potentiometer were used to read the temperature in the electric muffle furnace. It was found that the furnace temperature could be easily held at 500°±25° C. when the

[4] Kolthoff and Sandell (*9, pp. 329–331*) advocated repeated heating at 500° C. for 30-minute periods to constant weight. In order to test whether there has been decomposition of the calcium carbonate, they moisten the residue, or charge, with 2 or 3 drops of saturated ammonium carbonate solution, allow it to stand at room temperature for 15 minutes, dry it at 110° to 120° for half an hour, and weigh it. There should be no change in weight. If there is an increase in weight, the moistening is repeated with ammonium carbonate and the residue is heated until the weight is constant.

[5] Bau's precipitating reagent used 500 ml. of 50-percent acetic acid. A pH of 3.8 to 4.2, much lower than the pH given by the modified reagent, resulted.

proper rheostat setting was located. Meters that permit watching the temperature at all times are available, and there are still other devices that automatically maintain the furnace temperature at any desired point.

EXPERIMENTS TO TEST QUANTITATIVE ACCURACY OF IMPROVED METHOD

Several questions have arisen regarding the strictly quantitative nature of this method when applied to plant tissue under practical conditions of analysis. Is there complete extraction of oxalic acid from the tissue under the conditions chosen for the determination? Are there other relatively insoluble calcium salts present that would cause an error? Is the presence of barium, magnesium, and strontium a source of error? What is the effect of moderate pH changes on precipitation and subsequent recovery of oxalic acid? Does the solubility of the precipitated calcium oxalate seriously affect the accuracy of the final result? Does pectin interfere?

Completeness of Oxalic Acid Extraction

Dried ground rhubarb tissue was extracted with 1 liter of 0.25 N hydrochloric acid as described on page 37. Completeness of extraction of this material was then tested by adding a second liter of 0.25 N hydrochloric acid to the previously extracted residue and heating for 2 to 3 hours at 70° C. Negative results were obtained from both soluble and total oxalate determinations in a number of test cases with leaf-blade and petiole tissues which were relatively high in oxalic acid. The close agreement of replicate determinations shown in table 1 is further evidence of the uniformly thorough extraction of the material.

Effect of Other Insoluble Calcium Salts

Table 1 represents a sample record sheet carrying all the details of a typical series of analyses. The volume of the solvent (water or 0.25 N hydrochloric acid) and wash water was so great that other relatively insoluble calcium salts, such as calcium malate and calcium citrate, could be neglected as a contamination of the precipitate. These salts are sufficiently soluble to be removed almost completely by the method used.

Effect of Insoluble Salts of Oxalic Acid Other Than Calcium

Spectrographic examination was made of several calcium carbonate residues set aside from the analysis. Although small traces of magnesium, strontium, and barium were detected in the carbonate residue, the total of these elements would never be above 0.2 percent of the calcium carbonate charge. The residual calcium carbonate compared very favorably in purity with a chemically pure grade of calcium carbonate.

Effect of pH of Precipitation Medium

The effect of the pH of the precipitation medium on the precipitation and recovery of oxalic acid was determined as follows: A solution of oxalic acid the equivalent of 0.200 gm. of calcium carbonate (or

TABLE 1.—*Sample record sheet for total oxalic content of replicate samples of petiole tissue, 1932*

[All analyses reported in this study made by the modified and improved method described on p. 37]

Age of tissue part (days)	Total volume	Weight of sample	Sample in aliquot	Aliquot taken	Wash water	Calcium carbonate recovered	Oxalic acid equivalent	Anhydrous oxalic acid (dry-weight basis)		
								Sample	Average	Average deviation in relation to mean
	Milliliters	*Grams*	*Grams*	*Milliliters*	*Milliliters*	*Milligrams*	*Milligrams*	*Percent*	*Percent*	*Percent*
7	1,000	2.5	0.5	200	140	31.1	27.97	5.60	5.70	2.2
7	1,000	2.5	.5	200	100	31.0	27.88	5.58		
7	1,000	2.5	.5	200	55	31.1	27.97	5.60		
7	1,000	2.5	1.0	400	100	64.5	58.02	5.80		
7	500	1.25	1.0	400	60	65.5	58.96	5.90		
16	1,000	2.5	1.0	400	95	90.6	81.50	8.15	8.15	.0
16	1,000	2.5	1.0	400	70	90.6	81.50	8.15		
26	1,000	2.5	1.0	400	80	101.2	90.98	9.10	9.08	.27
26	1,000	2.5	1.0	400	80	100.7	90.54	9.05		
35	1,000	2.5	1.0	400	65	102.4	92.11	9.21	9.21	.0
35	1,000	2.5	1.0	400	50	102.4	92.11	9.21		
46	1,000	2.5	.5	200	100	61.4	55.23	11.05	11.07	.14
46	1,000	2.5	1.0	400	75	123.2	110.82	11.08		
60	1,000	2.5	1.0	400	75	135.2	121.6	12.16	12.37	1.7
60	500	1.25	1.0	400	45	139.7	125.7	12.57		
60 [1]	1,000	2.5	.5	200	110	68.6	61.71	12.34	12.21	1.1
60	1,000	2.5	1.0	400	120	134.3	120.8	12.08		
70	1,000	2.5	.5	200	100	78.9	70.97	14.19	13.98	1.0
70	1,000	2.5	1.0	400	65	154.8	139.2	13.92		
70	500	1.25	1.0	400	95	153.7	138.3	13.83		

[1] Second lot.

0.1799 gm. of oxalic acid) per liter was prepared in 0.25 N hydrochloric acid and treated with the precipitating reagent at pH 4.40, 4.75, and 5.05. Each hydrogen-ion concentration gave satisfactory precipitation from the standpoint of recovery of from 99.85 to 100.82 percent of the known concentrations, and the replicate determinations agreed reasonably well.

Effect of Solubility of Calcium Oxalate

Bau (*4*) precipitated calcium oxalate at a temperature not exceeding 7° C. in a cold room and gave the following values for the solubility: 1 liter of filtrate holds in solution the equivalent of 3.42 mg. of anhydrous oxalic acid; 1 liter of wash water holds in solution the equivalent of 4.64 mg. of anhydrous oxalic acid.

In order to determine the solubility of calcium oxalate under the conditions of these analyses, oxalic acid was recovered as before from 2 liters of a pure solution of sodium oxalate of known concentration. When the solubility values of calcium oxalate for the filtrate and the wash water were used in calculating the percentage of recovery, the recovery was found to be from 1.4 to 4.3 percent too high. When the solubility was neglected, however, the percentage of recovery was

found to vary only from 99.85 to 100.82 percent of the known value.

The technique followed in the use of the precipitant affords a possible reason why the solubility values need not be considered in this work. Bau's reagent always was allowed to stand several days in loosely stoppered bottles and then was filtered through a quantitative filter just before use. Bau (*3*) found that ordinary acetic acid may contain glyoxylic acid, which is oxidized by air to oxalic acid. Another source of oxalic acid as an impurity is the sodium acetate. Therefore, these two chemicals may contain enough oxalic acid to keep the reagent saturated with respect to calcium oxalate before it is used and this saturation tends to make the results too high. Furthermore, if the precipitate is washed rather rapidly with ice-cold water with the aid of suction and if the volume of washings and filtrate are kept to a minimum, at a temperature of about 5° C., the small amount of calcium oxalate dissolved is negligible. Calculations made of the ultimate solubility of $CaC_2O_4.H_2O$ in 100 ml. of water at 5° for three equivalent calcium carbonate recoveries, 25 mg., 50 mg., and 100 mg., amount to 1.64, 0.82, and 0.41 percent, respectively. Wash-water losses of calcium oxalate actually would be much lower than this because of the rapid washing with the aid of suction.

Effect of Presence of Pectin

It was difficult to get good calcium oxalate crystals from 0.5-percent pectin solutions, but concentrations of pectin in solution up to 0.4 percent did not influence the accuracy of the method under the conditions specified. The plant material under consideration contained no troublesome amounts of pectin when handled as described.

RESULTS OF ANALYSES

The 1932 samples (fig. 1 and table 2) show a pronounced increase

Table 2.—*Variation in oxalic acid content of rhubarb tissue with age, 1932*

Tissue and its age (days)	Total height of leaf	Total dry matter	Anhydrous oxalic acid			
			Soluble (in water)		Total (soluble in hydrochloric acid)	
			Fresh-weight basis	Dry-weight basis	Fresh-weight basis	Dry-weight basis
	Centimeters	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Leaf blades:						
7	13.9	16.87	0.14	0.83	0.24	1.42
16	32.7	12.84	.226	1.76	.299	2.33
26	49.8	11.50	.454	3.95	.542	4.72
35	63.0	10.95	.529	4.82	.637	5.82
46	83.5	11.73	.890	7.59	1.22	10.40
60	85.6	10.41	.960	9.23	1.40	13.47
70		11.04	.947	8.57	1.63	14.74
Petioles:						
7	6.0	6.00	.237	3.95	.342	5.99
16	17.7	4.95	.322	6.5	.403	8.15
26	27.8	5.74	.386	6.73	.521	9.08
35	36.6	5.53	.375	6.78	.509	9.21
46	46.6	6.64	.422	6.36	.734	11.06
60	44.6	6.71	.426	6.35	.830	12.36
70		6.75	.482	7.15	.944	13.98

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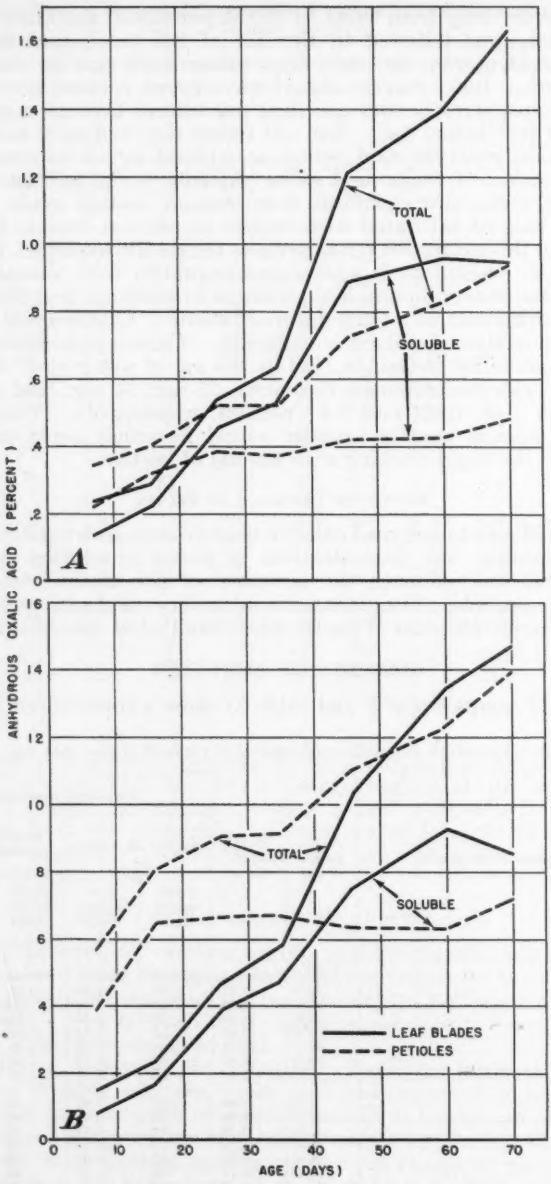

FIGURE 1.—Oxalic acid content of rhubarb leaf blades and petioles of different ages, 1932: *A*, Fresh-weight basis; *B*, dry-weight basis.

in concentration of total and soluble oxalates with age of the tissues. This situation is also shown to some extent for the soluble oxalate content in the 1935 series (table 3).

TABLE 3.—*Variation in soluble oxalic acid content of rhubarb tissue with age, 1935*

Age of tissue (days)	Total height of leaf	Total dry matter	Anhydrous oxalic acid in —			
			Leaf blades		Petioles	
			Fresh-weight basis	Dry-weight basis	Fresh-weight basis	Dry-weight basis
	Centimeters	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1	20.1	17.57	0.302	1.72	0.426	2.42
7	43.8	14.38	.311	2.16	.419	2.91
10	56.1	16.28	.496	3.05	.503	3.09
29	47.9	16.94	1.15	6.79	.523	3.09
35	47.5	14.76	1.22	8.26	.494	3.34
54	60.8	16.17	1.25	7.73	.561	3.47
65	62.3	15.46	.945	6.11	.572	3.70
70	72.0	15.52	1.02	6.57	.532	3.43

The petiole tissue up to 16 days of age showed a consistently higher concentration of both total and soluble oxalic acid than leaf-blade tissue (fresh-weight basis) in the 1932 series of samples and of the soluble acid in the young samples of the 1935 series (tables 2 and 3). When the stalks had reached full maturity (leaves 46 to 70 days old), the oxalic acid content of the petioles was about one-half that of the leaf blades for both total and soluble acid in all the determinations made. A narrower range in concentration of soluble and total oxalates is indicated for the petioles than for the leaf blades in all the material investigated. In the 1932 series, the mature leaf blades contained six or seven times as much oxalic acid as the young ones, whereas the petioles contained only two or three times as much.

The 1935 series (table 3) had roughly a content of 1.2 gm. of soluble oxalate per 100 gm. of fresh leaf-blade tissue for the 29- and 35-day samples. According to this analysis, one-half pound of leaf blades of either of these two samples would contain 2.7 gm. of soluble oxalic acid. It would take roughly a pound of petioles of somewhat older tissue (54 days or over) to contain an equivalent amount of soluble oxalate. Samples of rhubarb taken in other seasons immediately after a period of high photosynthetic activity might, of course, run higher in soluble oxalates than those shown in table 3. Van Itallie and Lemkes (*7*) found that the literature shows the fatal dose of oxalic acid to be 2 to 5 gm., but these writers thought that it was much lower. Kohman (*8*), by summing up much of the literature on this subject, found that the fatal dose ranges from 2 to 30 gm. He gave 10 gm. as the fatal dose for adults and 3 to 4 gm. for children.

DISCUSSION

Culpepper and Caldwell (*6*) made titratable-acidity determinations on rhubarb with as many as five series of samples taken as described herein. They found that the shape of the titratable-acidity curve

was in general the same throughout the season, differing only slightly in intensity in midseason or during the period of most active photosynthesis.

Previously no one had determined oxalates on such a series of samples having known ages and showing increasing stages in maturation. Other workers have analyzed rhubarb tissue for oxalic acid content, but they had not systematicaly investigated content in relation to age.

In this work Bau's procedure (*4*) was modified by ashing the calcium oxalate to the carbonate rather than to the oxide and certain improvements of technique which have given more reliable results than the older method were introduced. Although comparatively large volumes of extracting solution were used, experience has shown that smaller volumes can be used successfully. This means that it is possible to take, say, 2.0 to 2.5 gm. or any other suitable dry weight of sample, make it up to volume in a 250-ml. flask, and use two 100-ml. aliquots for the analysis if duplicate determinations are desired. Even the use of smaller volumetric flasks would serve for many problems when great sensitivity is not needed.

Aside from the error introduced by ashing the oxalate to the oxide, Bau's method of analysis has fallen into some disrepute because of certain ascribed sources of error supposedly inherent in the direct application to plant tissue. On the surface these supposed errors seem very important, yet when optimum conditions were found for carrying out the analysis there was no interference of any serious magnitude with the direct quantitative determination of oxalic acid in rhubarb tissue.

The most serious error which the procedure might possibly have would be due to the loss of oxalates upon improper drying of the fresh tissue that would favor enzymic changes in succulent material such as rhubarb petiole. In this work that possibility was minimized by slicing the material into thin sections and by the use of a fan-driven drier. Some analyses made of fresh tissue would suggest that a very slight loss had occurred in one or two cases with petioles, but other replicates were in good agreement.

Nearly all of the modern methods of analysis of plant tissue for organic acids employ ether extraction of the tissue as the first step; subsequently the separate organic acids, such as malic, citric, tartaric, and oxalic, are determined on the aliquots from the water extract of the ether residue. These methods are now highly refined and capable of yielding valuable data on acid metabolism in growing plants. Pucher, Wakeman, and Vickery (*13*) have perhaps done more for the development of sound analytical methods than any other group of workers in the organic acid field. Kohman (*8, p. 102*) stated: "There is great need for a reliable method for the accurate determination of oxalic acid in foods." It appears that when several organic acids must be determined the methods of Pucher et al. are the most reliable now available and should, therefore, be employed for detailed precision work. However, there are certain problems involving plants used as food, where there is a need for a less time-consuming method, specifically for oxalic acid, that is both reliable and reasonably accurate.

SUMMARY AND CONCLUSIONS

A direct and reliable modification of Bau's method (*4*) for the determination of oxalic acid in plant material by aqueous extraction of the ground dried tissue is described. In the use of this method it is important that succulent material be carefully prepared and dried under the proper conditions in order to avoid the enzymic loss of oxalates.

Certain sources of error believed by many workers to invalidate the usefulness of Bau's method have been investigated by the writer and found to be without appreciable influence on the quantitative recovery of oxalates from rhubarb (Ruby variety) tissue when suitable precautions in technique were adopted. Completeness of extraction of the tissue was assured by negative tests for oxalates when the residue was repeatedly extracted with hot water and dilute hydrochloric acid and by agreement of replicate determinations. Modifications of technique that make for reliability of the method were (1) ashing the calcium oxalate to calcium carbonate at $500° \pm 25°$ C. and weighing and (2) washing rapidly with ice-cold distilled water, using suction and a minimum volume of wash water and filtrate, which eliminates the need for taking solubility of calcium oxalate into account.

Pectin from rhubarb tissue and a purified commercial pectin in solution did not interfere with recovery; a relatively wide variation in pH (4.40 to 5.05) had no effect on the accuracy of the analysis.

The improved method was used to study the concentration of total and soluble oxalic acid in rhubarb leaf samples. These samples consisted of leaf blades and petioles of known ages; each sample of a particular age was composed of a minimum of 30 tagged individual leaves. Data were obtained representing equivalent anhydrous oxalic acid in water-soluble and hydrochloric-acid-soluble forms in average leaf blades and petioles of different known ages.

In the 1932 series the concentration of water-soluble oxalates of 26- to 46-day-old fresh rhubarb of market size was approximately 0.39 to 0.42 percent in the petioles and 0.45 to 0.89 percent, respectively, in the leaf blades. The 1935 series suggests what appears to be a seasonal march or increase in oxalates in the plant as the season advances. More analyses should be made on samples like the 1932 series taken three or four times during the growing season.

This study of the oxalate content of rhubarb leaves of different ages serves as an aid in determining the best time to harvest rhubarb for market. From these data it appears that rhubarb with leaves from 10 to 35 days old is best from the standpoint of eating quality and soluble oxalate content. However, there was not much increase in soluble oxalate in the edible stalk (petiole) of leaves 46 to 60 days old over that of those 35 days old.

The oxalate content of the leaf blade exhibited a more pronounced increase with age than that of the petiole. This was true of both soluble and total oxalates. The soluble oxalic acid in the petiole was initially higher in the young samples and went through only a very gradual increase as the petiole attained maturity. In the leaf blade the concentration of the water-soluble acid was lower initially, but at

full maturity (leaves 46 to 70 days old) its content was roughly double that found in the petiole on a fresh-weight basis.

In the 1932 series the concentration of both soluble and total oxalic acid was greater in the petiole tissue than in the corresponding blades for samples up to and including 16-day-old leaf blades.

The data obtained in this investigation support the idea expressed by Allsopp (*1*) and others that the continuous increase in acids in the leaf blades during the season of most active photosynthesis suggests that they arise either as a direct result of photosynthesis or indirectly from carbohydrates. The magnitude of the concentration changes alone in the leaf blades certainly makes it clear that the acid arises in a synthetic process involving photosynthesis.

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STAMEN MORPHOLOGY IN FLOWERS OF THE MUSKMELON[1]

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PREVIOUS OBSERVATIONS

The morphological interpretation of the dissimilar stamens which constitute the androecium of certain members of the Cucurbitaceae has been a controversial matter practically since the taxonomic description of the family was published. Recently, Heimlich (*4*)[2] described the androecium of cucumber (*Cucumis sativus*) as consisting of two complete stamens and one "half stamen" in which one theca (pollen sac) fails to develop, reaffirming the original interpretation of Naudin (*7*, *8*) that the tetrasporangiate stamens are normal, while the smaller bisporangiate stamens (half stamens) had resulted either from the abortion of the missing half or else from the splitting of a tetrasporangiate stamen in two. Judson (*5*) refers to only three staminodia in the carpellate flower of cucumber. Miller (*6*), comparing *Echinocystis lobata* with other genera, concluded that the bisporangiate stamen in Cucurbitaceae is a complete one and not a half organ in the developmental sense. He (*6*, *p. 277*) regards *Cucumis* as having "five stamens, four of which are united in two masses," and agrees with Payer (*9*, *pp. 440–446*), Eichler (*3*), and Baillon (*1*, *2*), who consider the tetrasporangiate stamen as a double organ. Miller (*6*) based his interpretation upon the observation that the tetrasporangiate stamen in *Echinocystis* has its origin in two separate primordia which form a single structure owing to the lateral coalescence and the growth of intervening tissue between the original primordia, and on the further fact that staminodia in the carpellate flower, although disposed like stamen primordia, commonly develop without combining in pairs. Thus, the members of the genus *Cucumis* are generally considered to be trimerous, at least in respect to the androecium, and according to Rosa (*10*), this condition is usually accompanied by a tricarpellate ovulary surmounted by a three-lobed stigma. Rosa (*10*) found two varieties of melons, the Golden Beauty casaba (*Cucumis melo* var. *inodorus Naud.*) and Pomegranate (*C. melo* var. *odoratissimus Naud.*) to be pentamerous both as to androecium and gynoecium. There are five single stamens (like the half stamen of other Cucumi) in both the staminate and perfect flowers, and there are five separate locules in the ovularian cavity.

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[2] Italic numbers in parentheses refer to Literature Cited, p. 54.

Most of these investigations have been made on plants of a monoecious character. In monoecious species, the carpellate flowers frequently bear staminodia but only rarely do these structures become functional stamens. Some genera have staminodia in the carpellate flower equal in number to the stamens in the staminate flower; other genera have staminodia in the carpellate flower unequal to the stamens in the staminate flower.

Almost all varieties of commercially cultivated muskmelons (*Cucumis melo*) show a modification of the monoecious characteristic which is termed andromonoecious. Andromonoecious plants bear perfect flowers in addition to carpellate ones and staminate ones. Rosa (*10*) found several exotic varieties of European and Asiatic origin which produced carpellate and staminate flowers but no perfect flowers. On the andromonecious plants of some varieties of *C. melo*, occasional carpellate flowers have been observed. The occurrence of these aberrant sex forms seems to be more common on plants cultured in the greenhouse. Rosa (*10*) observed 15 instances of carpellate flowers in 2,802 apparently perfect flowers, or 1 in 187, indicating that about 1 plant in 3 may produce a single flower of aberrant sex form. He could not correlate the occurrence of aberrant forms with any particular environmental condition, or with any certain stage in plant development.

In muskmelon breeding it is frequently necessary to emasculate the stamens from the perfect flowers. This procedure necessitates the examination of many flowers. In several commercial varieties and certain selections from inbred lines, variation from the usual "two complete and one half stamen" situation has been frequently noted in perfect flowers. Occasionally five bisporangiate individual stamens are observed. More frequently three bisporangiate and one tetrasporangiate stamen comprise the androecium. Such variation may likewise be observed in staminate flowers, though not so commonly. According to Rosa (*10*) most present-day varieties of melons present a trimerous gynoecium; however, both canteloups and watermelons occasionally produce fruits with four carpels. These observations suggested that the trimerous androecium of muskmelon flowers, which is now the prevailing type in most varieties, probably arose from the pentamerous by two bisporangiate stamens having united to form the tetrasporangiate stamen which seems to be a double organ. A study of the vascular anatomy of staminate and carpellate flowers was made to aid in the proper interpretation of the stamen arrangement.

STAMEN ARRANGEMENT

The androecium of the staminate flower of muskmelon usually consists of two groups of paired stamens and a single stamen. The paired stamens have, in each case, two vermiform or **S**-shaped thecae or "pollen sacs," but the single stamen has only one. Each theca results from the dehiasence of two mircrosporangia. The paired stamens adhere to each other by their connectives which prolong above the stamens. These prolongations are forked, indicating the double characteristic of the structure. The single stamen has only a single prolongation of the connective. Where this arrangement is observed

usually only three short filaments are apparent. The filaments diverge from the receptacle at the base of the inner surface of the perianth tube.

The stamen arrangement and development in the perfect flower may be the same as in the staminate flower, but frequently differs from the usual situation in the staminate flower and constitutes a clue to the homology of the individual stamens. Perfect flowers have been observed which contained five bisporangiate stamens. It seems obvious that the tetrasporangiate and bisporangiate organs cannot be homologous, for while one is a simple stamen, the other two, being made up of two single stamens, are compound.

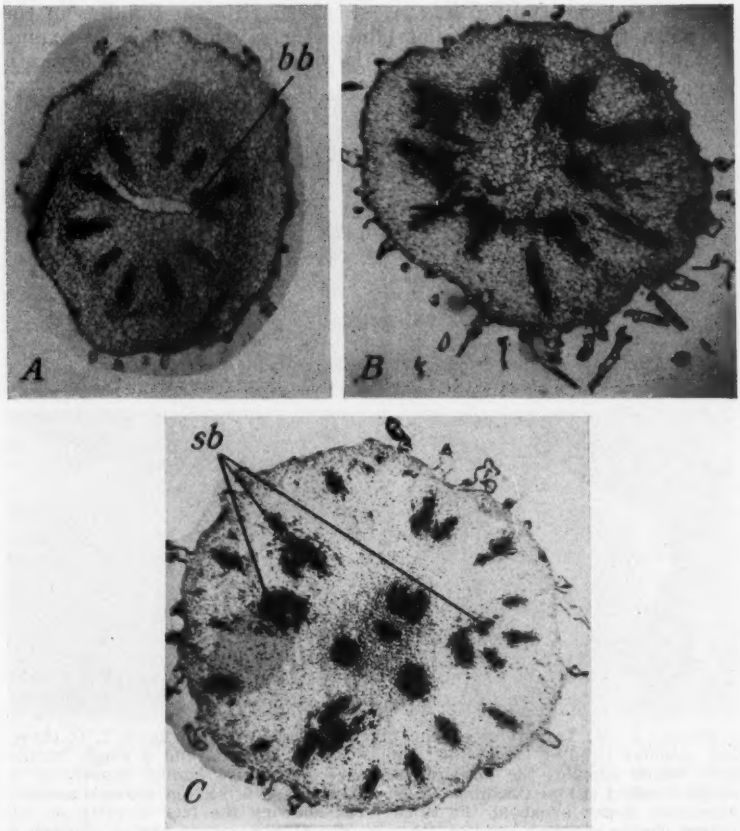

FIGURE 1.—*A*, Transverse section through a pedicel showing 10 main vascular bundles; 1 bundle has branched (*bb*). × 50. *B*, Transverse section through the receptacle of a staminate flower just above the vascular plate, showing 2 cycles of vascular bundles that traverse the perianth tube. × 36. *C*, Transverse section taken 240 μ nearer the perianth tube than *B*. Centripetal branches (*sb*) from bundles which supply the perianth extend to the stamens. × 41.

The vascular structure of the staminate and perfect flowers was investigated with particular reference to the vascular supply of the stamens.

VASCULAR CONTINUITY

The flower pedicel is traversed by 10 main vascular bundles (fig. 1, *A*). Before the pedicel joins the receptacle several bundles may branch, which usually results in 12 bundles with anastomosing branches which form a vascular plate at the base of the receptacle cup. From this plate usually 10 main bundles ascend obliquely through the receptacle and supply the perianth (fig. 1, *C*). These bundles, retaining their identity, extend outward and upward toward the sepal and petal lobes (fig. 1, *B*, *C*.). Between adjacent bundles in the perianth tube usually smaller bundles arise which likewise extend toward the petals and sepals from the vascular plate. In the perianth

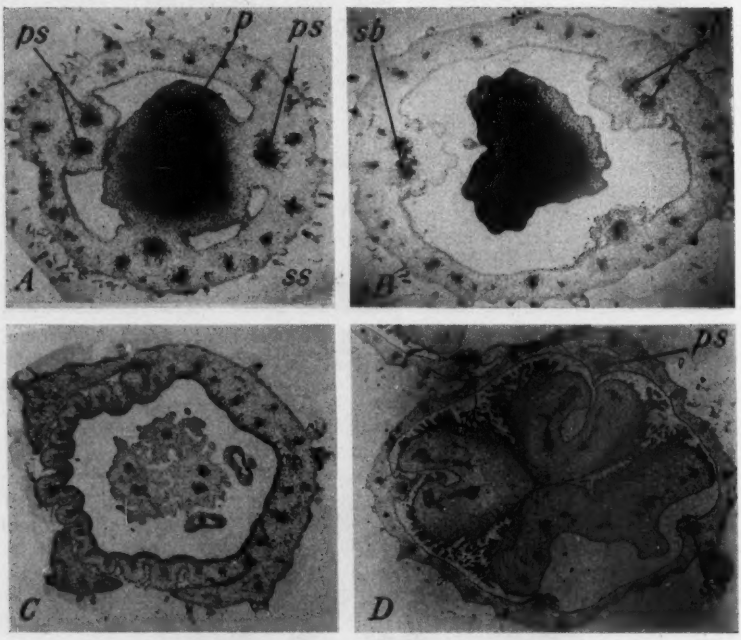

FIGURE. 2.—*A*, Transverse section taken about 250 μ from figure 1, *C*, showing vascular bundles (*ps*) which supply paired stamens and a single bundle (*ss*) which supplies the unpaired stamen; one double bundle appears as a single bundle; (*p*) pistillodium. × 34. *B*, Transverse section through another staminate flower at about the same level, showing the true identity of the paired double vascular bundles (*sb*). × 36. *C*, Transverse section through a staminate flower just above the stamens, showing the vascular bundles that extend through the prolongations of the connectives. × 26. *D*, Transverse section through a carpellate flower which contained three bisporangiate stamens containing a single bundle each and one tetrasporangiate stamen. The vascular bundles (*ps*) supplying the paired stamens appear like a single bundle; *ss*, a single bundle × 14.

tube the main bundles form two cycles (fig. 1, *B*, *C*). The bundles of the outer cycle terminate in the sepal lobes and alternate with the bundles of the inner cycle. Centripetal branches from the bundles of the inner cycle supply the stamens, while the main bundles lead to the petal lobes (fig. 1, *C*).

As shown by Heimlich (*4*) and Judson (5) in cucumber, and by Miller (*6*) in *Echinocystis lobata*, the sepal primordia and petal primordia arise in different cycles. Where double stamens occur in the androecium of *Cucumis melo*, each of the individual stamens arises in separate primordia, and the vascular bundles traversing these stamens may be traced to adajacent bundles within the cycle (fig. 1, *C*, and fig. 2, *A*, *B*). In transverse sections, the bundles supplying paired stamens sometimes appear as a single bundle (fig. 2, *A*, *D*). Where the vascular elements are so closely united, it appears that they may branch from adjacent bundles of the different cycles in the perianth tube (fig. 1, *C*, and fig. 2, *A*, *B*) ; i. e., from adjacent bundles supplying members of the calyx and corolla. When the stamens are not paired and each stamen retains its individuality in the androecium they are homologous and their vascular continuity may be traced to vascular bundles of the inner cycle in the perianth tube (fig. 2, *D*).

Each stamen is supplied by a single bundle (fig. 1, *C* and fig. 2, *A*, – *D*). The bundles are unbranched except near the distal end of the stamen where the branches extend to the microsporangia. Each bundle continues throughout the prolongation of the connectives. (fig. 2, *C*).

DISCUSSION

Miller (*6*) has shown that the tetrasporangiate stamen in *Echinocystis lobata* has its origin in two primordia that are distinctly separate and he concludes that this structure is double. Baillon (*1*) and Payer (quoted by Baillon) describe the same condition in *Bryonia*, indicating that the larger stamens are double.

The common occurrence of three and sometimes five individual bisporangiate stamens in *Cucumis melo* strongly suggests the double nature of tetrasporangiate stamens. The carpellate flower of *Fevillea*, according to Baillon, has five staminodia which correspond to the five bisporangiate stamens in the staminate flower. *Bryonia* also has five staminodia in the carpellate flower, but only three staminate masses in the staminate flower. As shown by Miller (*6*), *Echinocystis* reveals a similar situation—two staminate masses (three stamens) in the staminate flower and three staminodia of which two are paired, in the carpellate flower. *Thladiantha*, as described by Tison (quoted by Baillon) clearly shows the homology between the five separate bisporangiate stamens, but four of them are arranged in two pairs and although not united, stand close together. The fifth is isolated without a mate. This condition is not very different from that in *C. melo*.

While one may agree with Heimlich (*4*) that the number of vascular bundles in an organ cannot be regarded as proof of its homology, the presence of two bundles in each tetrasporangiate of *Cucumis melo* can hardly be interpreted in any other manner.

The bisporangiate condition of individual stamens in *Cucumis* as contrasted with the usual tetrasporangiate stamens in most angiosperms cannot be given great consideration in settling homologies, for variation exists in different families. Miller (*6*) has pointed out that from what may be regarded as a single microsporangium in the stamen of *Lemna* there is a progression up to large numbers of microsporangia in the branched stamens of such forms as *Ricinus* and *Calothamnus*, and bisporangiate stamens is the common situation in the *Asclepiadaceae*.

On the basis of the evidence presented, it seems probable that the flowers of muskmelon (*Cucumis melo*) and perhaps all of the Cucurbitaceae were originally pentamerous throughout. However, most present-day varieties of melons present a trimerous androecium and, according to Rosa (*10*), a trimerous gynoecium.

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VARIATIONS IN THE CHEMICAL COMPOSITION AND INSECTICIDAL PROPERTIES OF THE YAM BEAN (PACHYRRHIZUS)[1]

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INTRODUCTION

After the toxicity of the seeds of the yam bean to several species of insects had been established (*3*)[3], studies were made on the taxonomy of the genus (*1*) and on the chemical components of the typical species (*Pachyrrhizus erosus* (L.) Urban) (*6*, *7*). In the present paper some of the relationships between these various phases of the problem are reported.

MATERIALS

The samples upon which this study is based were collected in Mexico, Guatemala, and El Salvador during the late summer of 1943, or were sent to the laboratory for insecticidal and chemical evaluation by various individuals or agencies interested in the potential insecticidal value of the yam bean.

A detailed description of the various species is found in the taxonomic report of Clausen (*1*). *Pachyrrhzus erosus* is the common yam bean cultivated widely in Mexico for its edible watery sweet tubers. The samples of this species represent different varieties from various soil types and elevations. *P. strigosus* Clausen is a small-seeded species, which fact together with its limited distribution, would probably restrict its economic value as a source of insecticidal material. *P. tuberosus* (Lam.) Spreng. is a South American species with large seeds and large leaves, tubers, and vines. *P. ahipa* (Wedd.) Parodi is also a South American species, cultivated in Bolivia and in northern Argentina. It has plump seeds similar in shape to those of *P. tuberosus* but smaller. Material of the other two known species, *P. panamensis* Clausen and *P. vernalis* Clausen, has not been available in sufficient quantity for insecticidal and chemical analyses.

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[2] The authors are indebted to G. J. Goble and to Virginia Harley for assistance in the insecticidal and the chemical testing respectively. They are also indebted to the Office of Foreign Agricultural Relations and the Division of Plant Exploration and Introduction of the U. S. Department of Agriculture for aid in the collection and importation of seeds into the United States.

[3] Italic numbers in parentheses refer to Literature Cited, p. 64.

CHEMICAL METHODS

The samples were ground in a laboratory Raymond mill, extracted with chloroform, and the amount of oil and resin determined in the extract. Fluorescence measurements and several quantitative colorimetric tests proposed for rotenone determination were made on the resin fraction. Similar tests on the seven pure compounds previously isolated (7) showed sufficiently different responses (table 1) to permit estimation of the approximate amounts of these compounds in each sample.

The extractions and separations of oil and resin fractions were made on single samples. All color tests were made in duplicate on aliquots from the resin fractions. If duplicates failed to check within about 5 percent, further pairs of aliquots were run. If results were still inconsistent, or were unusually high or low for one or more of the constituents, a fresh sample was extracted and the complete analysis repeated. Where a color test showed an unusual intensity, it was repeated at a different dilution to bring the light transmission into a more reliable range (30–80 percent).

TABLE 1.—*Results of colorimetric, fluorometric, and insecticidal tests of pure compounds isolated from the yam bean* (7)

Test	Compound No.						
	I	II	III	IV (rotenone)	V (erosone)	VI	VII (pachyrrhizid)
Goodhue test (520 mμ)[1]	0	20	0	100	20	0	0
Meijer test (520 mμ)[1]	0	100	37	100	100	18	83
Meijer test (430 mμ)[1]	0	136	133	100	126	86	122
Rogers-Calamari test (590 mμ)[1]	0	107	0	100	136	0	24
Fluorescence (365-mμ filter for exciting light)	Intense; maximum at 410 mμ.	Slight	Intense; maximum at 480 mμ.	Slight	Slight	Slight	Slight
Toxicity to Mexican bean beetle	None	None	None	Toxic	Slight	None	Slight
Toxicity to silkworm	Slight?	Slight?	Slight?	Toxic	Toxic	None	Toxic

[1] As percent rotenone giving equivalent color.

EXTRACTION AND SEPARATION OF OIL AND RESIN

A 5-gm. sample of coarsely ground yam beans was extracted in a Soxhlet extractor with chloroform for 24 hours, the defatted marc dried and reground in a glass mortar, and the extraction continued for an additional 24 hours. Most of the solvent was distilled from the extract, and the remainder was removed by warming under reduced pressure until all foaming ceased.

The residue was taken up in 25 ml. of petroleum ether and 10 ml. of nitromethane, shaken in a separatory funnel, and the lower nitromethane layer drawn off. The petroleum ether layer was extracted with two more 10-ml. portions of nitromethane. The combined nitromethane extract was washed with one portion of fresh petroleum ether, which was added to the original petroleum ether solution.

The nitromethane solution was filtered, concentrated to a small volume under reduced pressure, and the last of the solvent removed by warming under a higher vacuum. The residue was weighed as the

resin fraction. The petroleum ether solution was treated similarly, and the residue weighed as the oil fraction. Heating was kept at a minimum during the removal of the solvent to avoid decomposition.

A light-brown insoluble powder appeared during the separation, and was removed by filtration of the fractions. It was discarded, since it showed no toxicity. The amounts of oil and resin extracted by several different solvents were substantially constant, whereas the amounts of this inert powder varied widely with the solvent. For this reason a direct weighing of the original total extract was not considered significant, and was omitted.

No further tests were made on the oil fraction, which showed no notable toxicity or response to color tests. The resin fraction, which contained practically all of the toxic material, was dissolved in acetone and diluted with acetone to 50 ml. Ten ml. of this solution was further diluted to 100 ml. with acetone to give the "stock solution" used for all of the subsequent tests.

FLUORESCENCE MEASUREMENTS

The acetone was removed from 0.2 ml. of the stock solution by warming under reduced pressure. The residue was dissolved in 10 ml. of redistilled benzene. Standards were prepared containing 0.1 microgram of compound I per milliliter of benzene and 1.0 microgram of compound III per milliliter of benzene respectively. A Coleman Photofluorometer with a 365-mμ filter for the exciting light and a 410-mμ filter for the fluorescent light was adjusted to read 0 for a benzene blank and 100 for the compound I standard. The compound III standard and the unknowns were then read. With the sensitivity setting left at the same point, the 410-mμ filter was replaced by a 480-mμ filter, the benzene blank again set to 0, and both standards and the unknowns read again. Any unknown showing a fluorescence stronger than the standards was diluted with a known volume of benzene, because the direct proportionality between fluorescence and concentration no longer held at higher concentrations. Under the specified conditions, the fluorescence given by each of the two compounds was directly proportional to its concentration, and the relative intensity at the two different wave lengths varied sufficiently to permit separate calculations of the concentrations of compounds I and III.

The equations for these calculations were derived from the standardization as follows: where F_{410} and F_{480} represent the numerical readings on the fluorometer scale with the two filters designated, and the symbols (I) and (III) represent the percentages of compounds I and III respectively in the original sample:

Standardization:	γ/cc.	F_{410}	F_{480}
Compound I	0.1	100	1.5
Compound III	1.0	45	30

With the sample and aliquot used, the percentage of each constituent in the original material is numerically equal to one-half the number of micrograms per cubic centimeter found in the aliquot, so that

$$F_{410}=2{,}000\ (I)+90\ (III) \quad (a)$$

$$F_{480}=30\ (I)+60\ (III) \quad (b)$$

Multiplying (b) by 1.5 and subtracting,

$$F_{410}-1.5\ F_{480}=1{,}955\ (I)$$

$$(I)=.00051\ (F_{410}-1.5\ F_{480}) \quad (c)$$

and from (b)

$$(III)=.017\ (F_{480}-30\ (I)) \quad (d)$$

Equations (c) and (d) were used for the final calculations, with the constants adjusted where necessary for variations in the standardization data.

MEIJER TEST [4]

The original Meijer test (*5*) was modified by the use of an acetone solution directly without dilution with water. A 0.5-ml. portion of the stock solution was pipetted into a 22-mm. matched test tube and 15.5 ml. of a solution of 100 mg. of sodium nitrite in 1 liter of concentrated sulfuric acid was added from a special pipette, with constant shaking. The tube was immediately placed in a bath at 20° C. After 1 hour the colors were read against a blank prepared at the same time in a Lumetron model 402E photoelectric colorimeter with filters of both 520 mμ and 430 mμ. The "rotenone equivalent" for each wave length was determined from calibration curves prepared with known amounts of pure rotenone.

GOODHUE (*2*) TEST

A 3-ml. portion of the stock solution was pipetted into a colorimeter test tube. Three milliliters of a freshly prepared mixture of 1 volume of potassium hydroxide (40 gm. per 100 ml. of water) and 7 volumes of sodium nitrite (1 gm. per liter of 95 percent alcohol) were added, the tube shaken, and placed in the 20° bath. After exactly 5 minutes, 7.5 ml. of dilute sulfuric acid (1: 3) were added, and the tube was shaken well and replaced in the bath. After 45 minutes, 15 ml. of ether were added, and the tube was shaken vigorously and replaced in the bath. Fifteen minutes later, the color in the lower layer was read against a blank developed for the same length of time, in the Lumetron colorimeter with a 520-mμ filter. The "rotenone equivalent" was determined from a calibration curve prepared with pure rotenone.

ROGERS-CALAMARI (*3*) TEST

Although Jones (*4*) reported that the form of the Rogers-Calamari test carried out in acetone solution did not appear to give good quantitative results, preliminary tests showed fully as good results as the other forms if special precautions were taken to keep times and temperatures strictly constant, to keep the solutions from strong light, and to compare the colors with blanks developed for exactly the same time. The latter was especially important, as the blanks change rapidly with time.

A portion of the stock solution was diluted with an equal volume of acetone, and a 5-ml. portion of the diluted sample pipetted into a colorimeter test tube. Five ml. of a 10-percent solution of thymol in acetone was added, and the tube left for 15 minutes in the 20° bath.

[4] Sometimes referred to as the Meyer test.

Then 0.1 ml. of 3-percent hydrogen peroxide and 5 ml. of concentrated hydrochloric acid were added as rapidly as possible, and the tube shaken for exactly 1 minute and replaced in the bath. Exactly 45 minutes after the addition of the acid, the color was read against a blank developed for the same time, in the Lumetron colorimeter with a 590-mμ filter. The "rotenone equivalent" was determined from a calibration curve prepared with pure rotenone.

CALCULATION OF INDIVIDUAL COMPOUNDS

The calculation of the quantities of compounds I and III from the fluorescence data has already been described. Calculation of the other compounds was made from the colorimetric data, based on their different responses to the various color tests as shown in table 1. The simplifying assumption was made that the contribution of compound VII to the Rogers-Calamari test was negligibly small. The colorimetric response of compounds II and V was too similar to permit separate determination, and average values were assumed for their colors and only their sum calculated. The following equations were derived for the calculations, where M_{520} and M_{430} represent the rotenone equivalents of the unknowns in the Meijer test at the designated wave lengths, G that in the Goodhue test, and R that in the Rogers-Calamari test, all in percentage of the original beans. The Roman numerals represent the percentage of the designated compound in the original beans.

I Determined by fluorescence
III Determined by fluorescence
Then, from table 1,

$$R=1.2\ (\text{II}+\text{V})\ (\text{avg.})+(\text{IV})+0.24\ (\text{VII}) \qquad (a)$$

$$G=0.2\ (\text{II}+\text{V})+(\text{IV}) \qquad (b)$$

Subtracting (b) from (a), and neglecting the contribution of VII,

$$(\text{II}+\text{V})=R-G \qquad \text{Equation 1}$$

Rearranging (b),

$$(\text{IV})=\text{G}-0.2\ (\text{II}+\text{V}) \qquad \text{Equation 2}$$

From table 1,

$$M_{430}=1.31\ (\text{II}+\text{V})\ (\text{avg.})+1.33\ (\text{III})+(\text{IV})+0.86\ (\text{VI})+1.22\ (\text{VII}) \qquad (c)$$

$$M_{520}=(\text{II}+\text{V})+0.37\ (\text{III})+(\text{IV})+0.18\ (\text{VI})+0.83\ (\text{VII}) \qquad (d)$$

Eliminating (VII) from (c) and (d) and solving for (VI),

$$(\text{VI})=1.67\ M_{430}-2.45\ M_{520}+0.27\ (\text{II}+\text{V})-1.32\ (\text{III})+0.78\ (\text{IV}) \qquad \text{Equation 3}$$

Solving (d) for VII,

$$(\text{VII})=1.20\ [M_{520}-(\text{II}+\text{V})-0.37\ (\text{III})-(\text{IV})-0.18\ (\text{VI})] \qquad \text{Equation 4}$$

SOURCES OF ERROR

The most serious source of error was the unavoidable omission of the unknown constituent probably present in the resin (*7*) which may account for a substantial part of the toxicity. Since this substance was not isolated in a pure state, its response to the color tests could not be determined.

Neglect of the Rogers-Calamari color given by compound VII would lead to high values for II+V and slightly low values for IV.

The values for VI and VII are considerably less certain than the others. Their calculation involves the results of several independent measurements, with a consequent accumulation of errors. In addition, the values for VI are probably consistently high because of the brownish color given in the Meijer test by some of the heterogeneous fractions. This color would mainly affect only compound VI because the latter is characterized by its yellow color in the Meijer test.

The calculations depend upon the assumption that the fluorescence and color tests given by each of the compounds are not affected by the others. The absence of large effects was established by preliminary tests, but detailed proof was impossible with the quantities of material available.

INSECTICIDAL TESTING METHODS

In making the insecticidal tests a representative sample of the coarsely ground beans was reground through the finest screen in the laboratory Raymond mill using 1 part of bean to 4 parts of Eastern Magnesia Talc (Emtco No. 23). This made a fine, rather oily dust which was used in all insecticidal tests. Since the dust was too oily to disperse well in a dusting tower, it was tapped out of a test tube through silk bolting cloth onto the larvae and leaves used in the test. Twenty-five third instar larvae of the Mexican bean beetle (*Epilachna varivestis* Muls.) were used in each test. Specimen dishes 110 mm. in diameter were lined with two thicknesses of moist paper toweling and a bean leaf was closely appressed to the bottom of the dish on the paper. The larvae were transferred to the test dish immediately before dusting. Mortality counts were made daily for 4 days. An insect was counted as alive if any movement was perceptible at the time of observation. No feeding occurred during the 4 days in any of the tests, and in nearly every case all insects were paralyzed so that the eventual kill with the concentrations and deposit used would be expected to be close to 100 percent. Four replicates were used in testing each material. Analysis of variance shows that highly significant differences exist between the samples tested.

RESULTS AND DISCUSSION

The source, species, chemical analysis, and toxicity to the Mexican bean beetle of each of the samples are presented in table 2.

CHEMICAL TESTS

The oil content of the different samples is strikingly constant. This fact is of interest because of the possible utilization of the oil as such, and because it eliminates the possibility of wide variations in physical or toxicological behavior of the different samples due to the effect of the oil.

The resin fraction, which contains practically all of the toxic components, shows considerable variation. The results of the color tests on the resin are roughly parallel to the quantities of resin, as might be expected.

The calculated quantities of the individual compounds also are roughly parallel to the resin content. The relative abundance of the compounds is approximately that expected from the quantities obtained in the work on isolation (7), with the exception of compound VI. This discrepancy may be accounted for both by the difficulty of isolating VI and by the interference in its colorimetric estimation already discussed under Sources of Error. The discrepancy is not serious, because of the lack of toxicity of VI. The somewhat erratic variation of compound I is not of great significance from the stand-

TABLE 2.—*Origin, chemical analysis, and toxicity to the Mexican bean beetle of yam bean samples* [1]

Sample	Origin	Altitude (meters)	Oil (percent)	Resin (percent)	Colorimetric values (percent rotenone equivalent)				Designated compounds calculated (percent)						Mortality in designated number of days (percent)			
					Meijer		Goodhue, 520 mμ	Rogers-Calamari, 590 mμ	I	II+V	III	IV	VI	VII	1	2	3	4
					520 mμ	430 mμ												
C-43-17	Ithaca, N. Y., greenhouse	250	25.7	1.5	0.60	0.99	0.26	0.50	0.018	0.24	0.10	0.21	0.28	0.07	4	6	33	37
C-43-77	Mexico	(?)	27.4	1.6	.85	1.38	.31	.56	.015	.25	.11	.26	.54	.29	8	35	57	67
C-43-82	El Salvador, Department of Santa Ana.	738	24.0	2.1	1.06	1.63	.32	.71	.013	.39	.12	.24	.46	.37	1	15	26	41
C-43-83	do	750	24.0	1.8	.96	1.52	.29	.65	.012	.36	.11	.22	.31	.34	16	49	66	84
C-43-84	El Salvador, Department of San Salvador.	710	26.3	2.4	1.22	1.86	.33	.77	.015	.44	.14	.24	.24	.54	7	38	60	73
C-43-88 [3]	Peru, Iquitos	(?)	22.4	1.6	.56	.95	.14	.24	.022	.10	.09	.12	.22	.32	2	27	43	56
C-43-98	Hawaii	(?)	28.4	2.8	1.37	2.13	.45	.84	.037	.47	.27	.32	.20	.40	14	43	50	79
C-43-99	Mexico	(?)	25.3	1.9	.89	1.36	.35	.58	.020	.23	.15	.30	.18	.32	4	31	59	71
C-43-100	do	(?)	26.5	2.1	.95	1.50	.36	.67	.024	.44	.18	.26	.24	.20	2	12	17	28
C-43-103	El Salvador	(?)	24.5	2.2	1.04	1.63	.32	.69	.013	.43	.15	.22	.24	.40	22	50	62	70
C-43-104	do	(?)	25.9	2.0	1.02	1.62	.30	.65	.009	.35	.11	.23	.33	.41	14	48	67	82
C-43-105	El Salvador, Department of San Salvador.	750	26.1	1.8	1.00	1.59	.31	.62	.011	.31	.11	.25	.33	.41	14	27	40	58
C-43-106	El Salvador, Department of Santa Ana.	750	25.7	2.2	1.31	1.94	.39	.82	.011	.43	.14	.30	.20	.58	2	32	56	84
C-43-107	El Salvador, Department of San Salvador.	710	25.7	2.0	.90	1.38	.34	.67	.011	.43	.09	.24	.26	.26	7	19	30	45
C-43-108	do	710	24.5	1.8	.98	1.50	.36	.77	.008	.41	.05	.28	.35	.25	26	41	60	73
C-44-20	Mexico, State of Guerrero	(?)	26.0	2.4	1.10	1.66	.45	.64	.015	.32	.11	.34	.23	.41	14	48	63	74
C-44-22	Florida, Dade County	5	22.5	2.4	1.00	1.97	.29	.64	.020	.35	.25	.22	.42	.19	10	29	44	55
C-44-23	Guatemala, Department of Santa Rosa.	1,218	25.6	2.2	1.06	1.70	.32	.74	.015	.42	.19	.24	.29	.33	5	18	33	39
C-44-24	Guatemala, Department of Zacapa.	225+	23.3	3.4	2.14	2.93	.84	1.46	.004	.77	.07	.55	.31	.81	27	61	80	84
C-44-41 [3]	Mexico, State of Chiapas	1,000±	19.6	2.6	1.07	1.70	.42	.92	.010	.50	.10	.32	.48	.14	6	28	55	77
C-44-42	Hawaii	(?)	26.2	2.5	1.07	1.88	.39	.94	.029	.55	.20	.28	.63	.19	6	28	50	68
C-44-46	Guatemala, Department of Cuilapa.	899	20.5	2.2	.95	1.46	.33	.84	.014	.51	.09	.23	.30	.14	25	45	57	72
C-44-47	Guatemala, Department of Santa Rosa.	1,218	21.3	3.3	2.02	2.90	.69	1.64	.006	.84	.43	.43	.12	.62	39	65	75	80
C-44-86	Mexico	(?)	24.0	1.8	.83	1.27	.29	.64	.022	.35	.15	.22	.15	.20	17	38	51	65
6061a	Mexico, State of Guanajuato	1,768	23.9	1.8	.70	1.13	.28	.49	.019	.21	.13	.24	.25	.18	13	36	44	73
6061b	do	1,768	24.4	1.8	.68	1.17	.23	.47	.020	.24	.12	.18	.33	.19	3	27	51	71
6061c	do	1,768	26.3	1.6	.82	1.26	.27	.63	.018	.36	.13	.20	.18	.22	23	45	55	62
6061d	do	1,768	25.3	1.8	.78	1.25	.29	.58	.020	.29	.11	.23	.28	.21	0	22	39	49
6062	Mexico, State of Oaxaca	1,526	23.6	1.8	.72	1.18	.25	.50	.021	.25	.11	.20	.30	.22	6	15	32	40
6063	do	1,526	26.2	1.8	.66	1.07	.19	.51	.017	.31	.13	.16	.20	.15	12	40	58	71
6096	Mexico, State of Chiapas	700	24.3	2.0	.95	1.52	.29	.65	.025	.36	.22	.22	.20	.30	0	12	20	29
C-44-158 [4]	Argentina, Buenos Aires	5													17	31	42	48

[1] Species *Pachyrrhizus erosus* unless otherwise designated. [2] *P. tuberosus*. [3] *P. strigosus*. [4] *P. ahipa*. Sample inadequate for chemical analysis.

point of insecticidal efficiency because this compound is nontoxic and has not yet been shown to have any relation to the other compounds. From the small quantity of compound V (erosone) originally isolated, and the relative abundance of II, it is very probable that the calculated values of II+V are made up largely of II. The values for IV (rotenone) are of great interest because of the toxicity of rotenone, and fortunately their determination is subject to relatively small errors by the present method. The values for VII (pachyrrhizid) are subject to considerable error, as already shown.

CORRELATION BETWEEN CHEMICAL AND INSECTICIDAL RESULTS

In the discussion of the chemistry of the yam bean (7) it was pointed out that of the various materials isolated, only rotenone and certain of the residues were toxic to the bean beetle. It was assumed, therefore, that a correlation might be found between the toxicity of different samples and the total resin or rotenone content or one of the colorimetric values. Any correlation with the quantities of the other compounds which might be found would be merely fortuitous. Correlation coefficients calculated between the toxicity and the analytical values are presented in table 3. The standard error of estimate in table 3 may be interpreted as the standard deviation of the number of bean beetle larvae killed in 4 days, as estimated from the chemical analyses.

The correlation coefficients are all significant, although not highly so. The standard error of estimate is high because of the many opportunities for experimental error in both the bio-assay and in the chemical analyses. The present results, however, establish as entirely practicable the use of one of the chemical determinations of table 3 to indicate those samples having a very high or a very low toxicity.

TABLE 3.—*Correlation coefficients and standard errors of estimate between 4-day mortalities of Mexican bean beetle larvae and analytical values of yam bean samples*

Analytical value	Correlation coefficient	Standard error of estimate
Resin fraction	0.376	15.9
Rotenone (IV)	.409	15.6
Meijer color (520 mμ)	.420	15.6
Meijer color (430 mμ)	.395	15.7
Rogers-Calamari color	.391	15.8
Goodhue color	.387	15.8

The correlation coefficients for the several determinations are not sufficiently different to establish a clear-cut choice of the best method. It was previously shown (7), however, that the silkworm is very susceptible to compound V (erosone) and VII (pachyrrhizid) as well as to IV (rotenone). Other things being equal, a determination sensitive to all three of these compounds should give the best measure of toxicity to a variety of insects. Neither the Goodhue nor the Rogers-Calamari test is given strongly by all three compounds, while all three are sensitive to the Meijer test. Accordingly, the latter is suggested as the most satisfactory measure of the toxicity of yam bean samples,

based on the information available at present. Measurement at 520 mμ is less subject to interference from compounds II, III, and VI, and is therefore preferable to 430 mμ.

MORPHOLOGICAL AND ENVIRONMENTAL FACTORS

The data in table 2 suggest that no correlation exists between morphological variation of the yam beans, their toxicity, and their content of rotenone, but more tests and analyses are necessary before this evidence should be regarded as conclusive. Since only one sample each of *Pachyrrhizus strigosus*, *P. tuberosus*, and *P. ahipa* were tested, information for these species is scarcely comparable with that for *P. erosus*, the most variable species, of which 29 samples were tested. In view of the variability in toxicity and chemical composition of *P. erosus*, the normal expectation is that *P. strigosus*, *P. tuberosus*, and *P. ahipa* will also prove variable in these respects, but none of these species exhibits the morphological diversity of *P. erosus*. The seeds of *P. strigosus* are the smallest known for *Pachyrrhizus*. In insecticidal value, they compare favorably with the best samples of *P. erosus*. The seeds of *P. tuberosus* are the largest found in the genus, but the sample from Peru must be classed with the poorest ones of *P. erosus*. The samples of P. *erosus* include the major morphological variations of that species. Seeds of collection No. 6061 were graded on the basis of size and shape as follows: (a) large round seeds; (b) small round seeds; (c) large square seeds; and (d) small square seeds. In insecticidal value the round seeds in this series were slightly better than the square seeds and the larger seeds of each shape were somewhat better than the small seeds. In content of rotenone, however, the square seeds averaged higher. This sort of discrepancy is evident throughout the data in table 2. Although nothing is known of the genetical details, the tentative conclusion is that no real correlation exists between morphological characteristics and presence of rotenone. Structural characteristics and chemical composition in this instance seem to vary independently.

The best insecticidal samples of *Pachyrrhizus erosus*, based on combined chemical and toxicological data, support this view. Sample C–44–24 came from plants with lobed leaflets (var. *palmatilobus*). The seeds were small and mostly square. Sample C–44–47 included plants with both unlobed (var. *typicus*) and lobed (var. *palmatilobus*) leaflets, a condition frequently encountered in wild or cultivated stands of *P. erosus*. The seeds averaged slightly larger and were more rounded than those of C–44–24. The insecticidal properties of two species, *P. panamensis* and *P. vernalis*, have not yet been studied. Conceivably, data for these might alter some of the above statements, but these species are probably all closely related genetically and possibly are similar chemically.

Possible correlation between geographical occurrence or habitat and content of rotenone were also investigated. The data concerning origin in table 2 show that the samples of *Pachyrrhizus erosus* were obtained from diverse localities scattered throughout the general range of the species, as well as from different altitudinal levels. Consideration of these data fail to suggest either that plants from any particular

altitudinal level are better insecticidally or that those from any part of the range of the species are superior. Two of the best samples came from different sides of Guatemala, C–44–24 from the Department of Zacapa, at 225 meters, and C–44–47, from the Department of Santa Rosa, at 1,218 meters. Another sample, also from the Department of Santa Rosa, gave very poor results in tests with larvae of the Mexican bean beetle, and was only average in rotenone content.

SUMMARY

The relationships between origin, chemical analyses, and insecticidal value of 31 yam bean samples have been studied in tests with Mexican bean beetle larvae. Significant correlations were found between toxicity and resin content, rotenone content, and three colorimetric analytical values. Neither the oil content nor six compounds other than rotenone isolated from the yam bean were significantly correlated with toxicity. The Meijer color test is proposed as a suitable chemical method for indicating approximately the toxicity of yam bean samples.

Morphological variation, geographical occurrence, and habitat have not been shown to affect the toxicity or composition of the sample. Three species of yam bean not previously studied, *Pachyrrhizus tuberosus* (Lam.) Spreng., *P. strigosus* Clausen, and *P. ahipa* (Wedd.) Parodi, are reported as toxic to the larvae of the Mexican bean beetle.

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